

Remarks

The Applicant confirms the earlier election of Group III including Claims 20, 22, 25 and 26. The Applicant has cancelled Claims 1-19, 21, 23 and 24 to expedite allowance. Those claims have been cancelled without prejudice and without disclaimer of the subject matter therein. The Applicant reserves the right to file one or more divisional applications directed to the subject matter of those claims.

Claims 20, 22, 25 and 26 stand rejected under 35 U.S.C. §112, first paragraph. The Applicant notes the Examiner's helpful comments with respect to "one halogenated compound" and "one N-halogenated derivative." Claims 20 and 22 have been amended to recite at least one hypochlorite compound and at least one N-chlorinated compound. Withdrawal of the rejection is respectfully requested.

Claims 20 and 22 stand rejected under 35 U.S.C. §112, first paragraph. The Applicant has removed reference to "preventing" from those claims. Withdrawal of the rejection is respectfully requested.

Claims 20, 22, 25 and 26 stand rejected under 35 U.S.C. §112, second paragraph as being indefinite. The Applicant has removed the term "derivative" and "or their derivatives." Withdrawal of that portion of the rejection is respectfully requested.

The Applicant has also amended Claims 25 and 26 in accordance with the Examiner's helpful suggestion. The "linked to" has been removed and substituted by "generated from." The Applicant respectfully submits that Claims 25 and 26 are now in compliance with §112, second paragraph. Withdrawal of the rejection is respectfully requested.

Claims 20, 22, 25 and 26 stand rejected under 35 U.S.C. §103 over Julich. The Applicant notes with appreciation the Examiner's detailed comments hypothetically applying Julich to those

claims. The Applicant respectfully submits that, however, Julich fails to teach or suggest the subject matter of those claims. Reasons are set forth below.

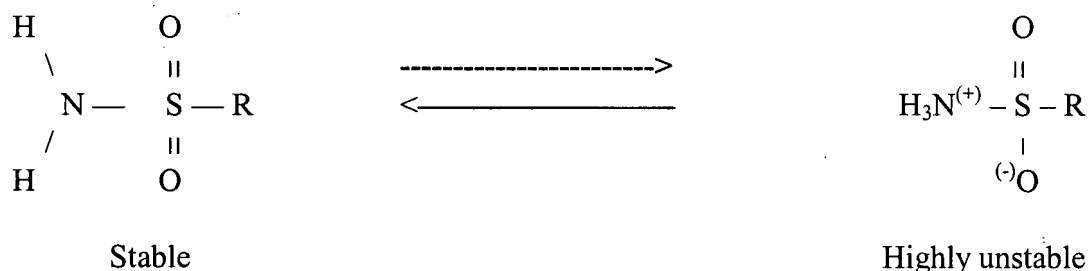
The Applicant agrees that Julich discloses chloramine T and sodium hypochlorite (NaOCl). The Applicant also agrees that Julich does not teach the combination of chloramine T and sodium hypochlorite for the treatment of infections. Nonetheless, the rejection takes the position that it would be obvious to combine the two in a single composition for the treatment of various infections.

The Applicant respectfully submits that there is no incentive provided by Julich to combine chloramine T and hypochlorite. It is merely speculative that there would be any benefit in so doing. This is essentially an “obvious to try” situation which was long ago banned by the Federal Circuit. On this basis alone, the Applicant respectfully submits that the claims are patentable over Julich.

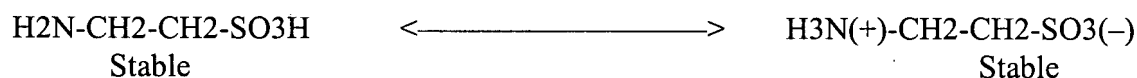
In any event, there is a further problem associated with Julich. This relates in particular to the disclosure of chloramine T. In that regard, it must be noted that the internationally accepted nomenclature for chloramine T is: N-chloro-*p*-toluenesulphonamide sodium. The Applicant invites the Examiner’s attention to the enclosed copies of Meyers and Fuersted where this is confirmed. On the other hand, the internationally accepted nomenclature for taurine N-chloramine is: N-chloro-2-aminoethanesulfonic acid ($\text{ClNH}_2\text{CH}_2\text{CH}_2\text{SO}_3\text{H}$). Also, chloramine T is a member of the sulphonamide family ($\text{R}_1\text{-S-N-R}_2$). In sharp contrast, taurine is a member of the sulfonic acid family ($\text{R}_3\text{-SO}_3\text{H}$).

The differences are further illustrated below with additional comments as follows. A zwitterion is a neutral compound that has an equal number of one unit formal electric charge and with opposite polarity. It is usually restricted to compounds with charges on non-adjacent atoms. Chloramine T is N-chloro-*p*-toluenesulphonamide sodium generated from the reaction between hypochlorite and *p*-Toluenesulfonamide molecules (with the following Stoichiometry: 1/1 molecule).

The Applicant invites the Examiner's attention to the enclosed copies of UMESC and Masten_Extracts 2002. *p*-Toluenesulfonamide is not a member of the amino acid family and is not included within the zwitterion family. It is due to the high proximity between the N(+) and O(-) atoms which gives this conformity huge instability because of the electronic conflict generated from the adjacent-like electronic structure:



In sharp contrast; Taurine is an amino acid with a highly stable zwitterionic structure. The absence of an adjacent-like electronic structure explains this high stability.



As a consequence, the Applicants respectfully submits that chloramine T as disclosed by Julich and the taurine N-chloramine as disclosed by the Applicant are two completely different molecules. Thus, even if one skilled in the art were to hypothetically formulate a composition utilizing both the NaOCl and chloramine T of Julich, the result would still fail to teach or suggest the Applicant's claimed subject matter of at least one hypochlorite compound and at least one N-chlorinated compound selected from zwitterionic compounds and/or the amino acids. Withdrawal of the rejection based on Julich is respectfully requested.

In light of the foregoing, the Applicant respectfully submits that the entire application is now in condition for allowance, which is respectfully submitted.

Respectfully submitted,

A handwritten signature in black ink, appearing to be 'T. Daniel Christenbury', written in a cursive style.

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PREPARATION AND CHEMICAL CHARACTERIZATION OF RADIOIODINATED BLEOMYCIN

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None of the radionuclides with which bleomycin has been labeled have chemical and nuclear properties that are entirely satisfactory for in vivo tumor localisation. Bleomycin has been radioiodinated by the iodine monochloride, chloramine-T, and lactoperoxidase methods. Iodine monochloride proved to be the preferred method and conditions were developed whereby 80% of radioiodide was covalently bound to bleomycin. Bleomycin (140 µg) was added to 200 µl of saline/citrate buffer (pH 7.0) followed by radioiodide and iodine monochloride. This reaction mixture was incubated for 1 hr and purified by Sephadex G-10 chromatography. The iodine monochloride reaction product underwent hydrolytic deiodination in vitro at a rate of about 1.2%/day (0.15 M NaCl, 37°C). Bleomycin A and B components were radioiodinated with equal efficiency on a mole fraction basis.

Bleomycin is a chemotherapeutic antibiotic that has been investigated as a potential tumor-localizing radiodiagnostic agent. It exists naturally as a copper (II) ligand (1) and it is this chelating ability that has been exploited for all of the isotopic labels reported thus far. Both divalent and trivalent ions that chelate bleomycin have been identified but divalent cations, especially Co^{2+} , form more stable bonds than trivalent cations such as In^{3+} or SnCl_2 -reduced TcO_2 (2).

Because of the disadvantageous nuclear properties of those radioisotopes that exist chemically as divalent ions (e.g., ^{64}Cu , ^{57}Co , ^{62}Zn) and the instability of higher valence complexes (e.g., ^{111}In , ^{67}Ga , $^{99\text{m}}\text{TcO}_2$ / SnCl_2) of bleomycin, we have investigated methods for labeling bleomycin with radioiodine. A covalent iodine-bleomycin bond is likely to be stronger than the metal-bleomycin coordinate bond

and, therefore, more resistant to hydrolytic breakdown or competitive displacement. Because ^3H -bleomycin containing no transition metal ions has been shown to clear faster and to be less toxic, yet have equivalent tissue distribution kinetics to Cu^{2+} chelated bleomycin (3), a nonchelated bleomycin can be expected to improve the tumor-to-background relationship and reduce the radiation dose. Therefore, ^{123}I -labeled copper-free bleomycin may be the ideal form for this radiodiagnostic agent. We report here a study of bleomycin iodinated by the iodine monochloride, chloramine-T, and lactoperoxidase methods.

MATERIALS AND METHODS

Bleomycin as the sterile copper-free sulfate Blenoxane® (Bristol Laboratories, Syracuse, N.Y.) was dissolved in distilled water to a concentration of 1 mg/ml. Chloramine-T (sodium-N-monochloro-p-toluene sulfonamide) was dissolved in distilled water to a concentration of 1 mg/ml and prepared fresh before each experiment. Lactoperoxidase (E.C. No. 1.11.1.7) was used as supplied by the Sigma Chemical Company, St. Louis, Mo. and was dissolved in 0.05 M phosphate buffer to a concentration of 1 mg/ml. Buffers used were 0.067 M phosphate, 0.10 M borate, and 0.15 M saline + 0.02 M citrate. All other chemicals were reagent grade and were not purified further.

Radioiodination yields may depend on such reaction conditions as reactant concentration, buffer identity, ionic strength, pH, and duration of reaction. In order to maximize yields, the following standard procedures were adopted. Some procedural varia-

Received Nov. 20, 1974; revision accepted April 11, 1975.
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tions and their effects will be described in the RESULTS section.

Iodine monochloride (4) labeling was accomplished by adding 140 μg (0.1 μmoles of bleomycin) to 200 μl of saline/citrate buffer. The radioiodine was added in a volume of 5–500 μl after which 30 μl of 0.0033 *M* iodine monochloride (0.1 μmoles), prepared according to the method of McFarlane (4), was added. The reaction mixture was shaken for 5 min and allowed to react for at least 1 hr before preparative separation. Aliquots of the reaction mixture were sampled every 5 min for 2 hr and electrophoresis performed to determine the effective reaction rate.

Chloramine-T labeling (5) was accomplished by adding 100 μg of bleomycin to 500 μl of phosphate buffer (pH 7.5), followed by the radioiodine and 25–100 μg of chloramine-T. The reaction was quenched after 5 min by adding 0.25 ml of sodium metabisulfite (1 mg/ml).

Lactoperoxidase labeling (6,7) was accomplished by adding 500 μg of bleomycin to 500 μl of phosphate buffer (pH 7.5); radioiodine was added followed by 10 μg of lactoperoxidase and finally 10 μl of 1 *mM* hydrogen peroxide. The mixture was incubated at 37°C for 40 min and quenched by the addition of sufficient cysteine to decompose the hydrogen peroxide.

The analytic separation of I-bleomycin, iodate, and iodide was by electrophoresis (340 V, 6 mA 10 min) on cellulose acetate strips (Sepraphore III, Gelman Instruments, Ann Arbor, Mich.) in sodium barbital buffer (0.05 *M*, pH 8.6). Under these conditions, I-bleomycin stays at the origin and iodate and iodide migrate 2.8 ± 0.8 and 5.8 ± 1.5 cm, respectively. This separation was also used to evaluate the radiochemical purity of *I-bleomycin following a preparative separation. All yields were based on the percent of radioiodine bound to bleomycin as determined by electrophoresis.

The preparative separation of I-bleomycin from I^- and IO_3^- was achieved by Sephadex chromatography. Typical retention volumes are given in Table 1. A Sephadex G-10-120 column was preferred for preparative separations because a shorter column could be used and A and B components of bleomycin are not fractionated. A Sephadex G-25-40 column was used to determine the relative labeling efficiency of the A and B components of bleomycin. Fractions were collected and the specific activity of each determined from spectroscopic (optical density at 280 nm) and radioactivity measurements and recorded in the arbitrary units of counts per minute per optical density. The yields from Sephadex

TABLE 1. ELUTION VOLUMES FOR SEPHADEX COLUMNS: 50 ML BED VOLUME ELUTED WITH 0.15 *M* SALINE

Elution volume	Sephadex G-25-40	Sephadex G-10-120
Void volume	18	30
I-bleomycin A	36	31
I-bleomycin B	43	31
IO_3^-	64	39
I^-	50	92

TABLE 2. BLEOMYCIN IODINATION YIELDS

Reaction	Buffer (pH)	Experiments (No.)	Yield
Iodine monochloride	Saline citrate (6.5)	3	60
	Phosphate (6.5)	3	65
	Saline citrate (7.0)	20	80
	Phosphate (7.0)	3	80
	Saline citrate (7.5)	3	75
	Phosphate (7.5)	3	75
Chloramine-T	Phosphate (7.5)	3	80
Lactoperoxidase	Phosphate (7.5)	4	10–22

were 95% or more of the electrophoresis yields. Dowex 1X-8 anion-exchange resin is effective in separating I-bleomycin from I^- but was not used when IO_3^- was present.

The stability of the I-bleomycin purified by Sephadex chromatography has been measured by incubating samples in 0.15 *M* sodium chloride solution at 37°C. Polystyrene test tubes were used, an aliquot was removed at daily intervals for a period of 1 week, and electrophoresis performed to determine the rate at which iodide was liberated.

RESULTS

Labeling chemistry. Labeling yields of bleomycin radioiodinated by the iodine monochloride, chloramine-T, and lactoperoxidase methods are given in Table 2. Both iodine monochloride and chloramine-T methods gave yields of 80% or more based on radioactivity measurements and depending on reaction conditions. Lactoperoxidase yields ranged from 10 to 22% and, therefore, this method of labeling was not pursued.

For the iodine monochloride method, labeling yields were highest between buffers of pH 6.5–7.5, and both phosphate and citrate buffers gave equivalent yields. Labeling efficiencies were unchanged when reaction volumes were varied between 0.5–4 ml. The yield of I-bleomycin progressively increased for 2 hr when iodine monochloride was used but was 60% and 75% of the maximum value at 5 and

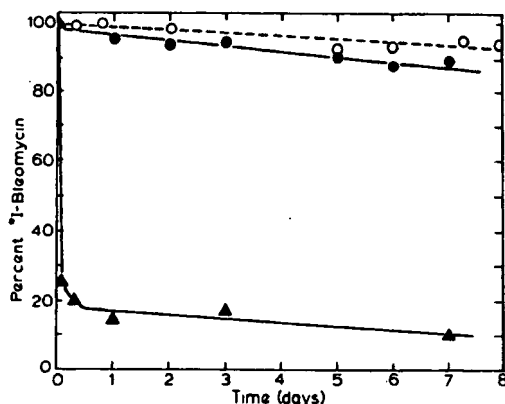


FIG. 1. In vitro hydrolysis of ^{125}I -bleomycin. Timing was begun immediately following preparation and purification (Sephadex G-10), and percent ^{125}I -bleomycin was determined by electrophoresis. Hydrolysis conditions: 0.15 M saline, 37°C. Labeling by iodine monochloride (● and ○ represent two different experiments using the same methods) and chloramine-T (▲). Regression lines --○-- , $Y = 99.7 - 0.84 T$, $r = 0.903$ and --●-- , $Y = 97.8 - 1.48 T$, $r = 0.924$.

15 min, respectively. The minimum incubation time used for preparative labeling was 1 hr. Optimum labeling efficiency and specific activity of greater than 100 mCi/mg were obtained with 0.14 mg (0.1 μmoles) of bleomycin to which was added 10 mCi ^{125}I -NaI and 30 μl of iodine monochloride.

The chloramine-T reaction yield did not increase with extended incubation time; nevertheless, chloramine-T labeling resulted in a higher specific activity product than iodine monochloride. Due to product instability (see next section), the effects of different reaction conditions on labeling efficiency were not studied further.

In three different experiments, Sephadex G-25 chromatography of ^{125}I -bleomycin prepared by iodine monochloride showed equal specific activity for both A and B components. In a typical experiment, the product yields were 18,756 cpm and 8,348 cpm and 0.036 o.d. and 0.016 o.d. for the A and B components, respectively. This corresponds to specific activities (radioactivity/mass \approx cpm/o.d. of 5.21×10^5 and 5.22×10^5 for the A and B components. The relative concentration of A and B was 69% and 31% of the total bleomycin, respectively, and was constant for the bleomycin which we used.

Stability. The stability of ^{125}I -bleomycin labeled by the iodine monochloride and chloramine-T methods is shown in Fig. 1. Iodine monochloride-labeled bleomycin undergoes hydrolytic deiodination at a rate of $1.16 \pm 0.32\%$ /day. This is in contrast to the chloramine-T reaction product where 80% is hydrolyzed in the first 8 hr followed by about 1%/day deiodination for the remaining 20% of the product.

DISCUSSION

None of the radionuclides with which bleomycin has been labeled have chemical and nuclear properties which are entirely satisfactory for in vivo tumor localization. Covalent labeling of bleomycin with ^{125}I might provide a tracer bleomycin with a nearly ideal combination of nuclear, chemical, and biologic characteristics for scintigraphic imaging of patients with neoplastic disease.

Iodination of bleomycin by the chloramine-T method was first described by Renault, et al (8) but they abandoned it because the product deiodinated in 0.1 M phosphate buffer at a rate of 5%/day. We found the chloramine-T reaction product to be even more labile. In the experiment shown in Fig. 1, 85% of the ^{125}I -bleomycin bonds was hydrolyzed in the first day. This is in contrast to the iodine monochloride reaction product where 1.2% was hydrolyzed each day. These observations suggest that iodine monochloride and chloramine-T cause radioiodine to be bound to bleomycin in different ways.

Lactoperoxidase is useful for radioiodination of large protein molecules containing tyrosine residues but is less effective for histidyl labeling (6,7). Bleomycin contains one β -hydroxyhistidine but no tyrosine residues whereas lactoperoxidase contains several tyrosine residues so that at least a 50-fold excess of bleomycin over enzyme must be used in the labeling procedure to minimize self-iodination of the lactoperoxidase. A minimum of 10 μg of enzyme is needed to catalyze the iodination reaction so that 500 μg of substrate bleomycin is required for each iodination. In contrast, the iodine monochloride procedure uses 0.14 mg bleomycin and, therefore, provides a higher specific activity product. From these considerations, we chose to concentrate on iodine monochloride as the agent for bleomycin iodination.

The iodine monochloride labeling yield reached a maximum of $80 \pm 5\%$ at pH 7.0–7.5 for the buffers studied. Whereas the iodine monochloride reaction with tyrosine-containing polypeptides is complete in a few minutes (4), the yield for bleomycin continued to increase during the first 2 hr of labeling. The modified histidine residue is the probable site of bleomycin iodination; therefore, the observed rate difference is consistent with the results of other workers who have shown that histidyl residues are less reactive toward electrophilic iodine than are tyrosyl residues (9).

The fact that neither the A nor B components of bleomycin are labeled preferentially leads us to believe that the terminal amine residues are not the site of iodination and further, that they do not influence the iodination reaction. The difference in these

two families of bleomycin fractions is that bleomycin A contains sulfur in the terminal amine residues but bleomycin B contains only amines (10).

Bleomycin radioiodinated by the iodine monochloride method is stable in vitro and, within the range of selectivity of the chromatography, bleomycin and ¹²⁵I-bleomycin were not distinguishable. The chromatographic methods separate bleomycin based on molecular size as well as on ionic charge properties, so that any lytic or oxidative side reactions caused by iodine monochloride were probably not significant. From our chemical studies, it could, therefore, be anticipated that ¹²⁵I-bleomycin will behave in vivo like bleomycin. Studies in animals, however, will provide a more critical biologic test of this new radiodiagnostic agent. These studies are presently being performed and, although of a preliminary nature, appear promising.

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Southwestern Chapter SOCIETY OF NUCLEAR MEDICINE 21st Annual Meeting

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ANNOUNCEMENT AND CALL FOR ABSTRACTS

The Program Committee welcomes the submission of contributions in nuclear medicine from members and nonmembers of the Society of Nuclear Medicine for consideration for the program, including scientific, teaching, and technologist sessions.

Each abstract should:

1. contain a statement of purpose, methods used, results, and conclusions
2. not exceed 250 words
3. give title of paper and names of authors as you wish them to appear on the program. Underline the name of the author who will present the paper. Send the abstract and two copies to

Deadline: December 1, 1975

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Evaluation of bactericidal activity and lag of regrowth (postantibiotic effect) of five antiseptics on nine bacterial pathogens

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Lag of regrowth or postantibiotic effect (PAE) relates to suppression of bacterial regrowth following short exposure to an antimicrobial agent. A delay in regrowth has not yet been studied for antiseptics to any great extent. We therefore examined and compared the lag of regrowth and the bactericidal activity of five antiseptics (chloramine T, chlorhexidine, povidone-iodine, phenoxyethanol and mandelic-lactic acid) against nine bacterial pathogens. Delay in regrowth was determined by application of two concentration–time schedules: a test concentration at the MBC with a contact time of 1 h or using fixed suboptimum concentration of each antiseptic for 2 min (optimum concentrations sterilized the culture, impeding assessment of regrowth) followed by a neutralization–dilution step and subsequent viable counting to follow bacterial regrowth. Each antiseptic displayed a different spectrum of activity in terms of MIC or MBC, bactericidal effect and lag of regrowth. The delay in regrowth varied from 0 to 5.7 h with only a few discrepancies between the two treatment schedules. Mandelic-lactic acid and chloramine T induced a significantly longer lag as compared with the other agents, whereas phenoxyethanol produced the shortest lag values. No significant correlation between the killing rate and the lag of regrowth could be demonstrated. Information on bactericidal activity, as well as lag of regrowth, could be a useful screening method for the efficacy of antiseptics. Moreover, data on lag of regrowth could contribute to the choice of antiseptic and guide in determining the optimum interval between repeated applications of antiseptics.

Introduction

An important feature of an antibiotic's activity is the postantibiotic effect (PAE) or lag of bacterial regrowth following a short exposure of bacteria to an antimicrobial agent. Although a PAE has been demonstrated to be a feature of most antimicrobial agents against a variety of common bacterial pathogens¹ only a few papers have been published on lag of regrowth after treatment with antiseptics.^{2–4} Such data may contribute to the choice of antiseptic because an extended lag will induce an antibacterial effect longer than expected from the contact time. Moreover, a prolonged lag phase may allow an extended application interval for a given antiseptic. Thus, the aim of this study was to study and examine a potential lag of regrowth among antiseptics by testing five urinary antiseptic solutions (chloramine T, chlorhexidine, povidone-iodine, phenoxyethanol and a mixture of mandelic acid

and lactic acid).^{5–7} Two test regimens were applied: one regimen with a contact time of 1 h using equipotent concentrations (1 × MBC) and a regimen with a contact time of 2 min using fixed (suboptimum) concentrations of each antiseptic. The bactericidal activity of each antiseptic was also determined.

Materials and methods

Bacterial strains and media

The strains used in this study were either type strains (when available) or clinical isolates from our collection: *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25913), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter cloacae*, *Staphylococcus epidermidis* and *Serratia marcescens*. Strains were

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stored at -80°C and were subcultured on 5% blood-agar plates before testing. Bacteria were grown in Mueller-Hinton broth (MHB) supplemented with 20 mg/L Mg^{2+} and 50 mg/L Ca^{2+} (Statens Serum Institut, Copenhagen, Denmark) and viable counts were performed on 5% blood-agar plates (Statens Serum Institut). All studies (except the MIC or MBC determinations) used organisms from logarithmic-phase cultures. This log-phase inoculum was derived from an overnight broth culture dilution in MHB to the desired bacterial concentration and incubated at 35°C for 1–2 h before each experiment. Viable counts were performed immediately to verify the original inoculum.

Antiseptics and neutralizer

Chlorhexidine (chlorhexidine acetate solution 0.2% w/v) was obtained from Nycomed DAK (Copenhagen, Denmark), whereas povidone-iodine (polyvinyl pyrrolidone iodine complex powder), phenoxyethanol (ethyleneglycolmonophenylether phenylglycol powder), chloramine T (*N*-chloro-*p*-toluensulphonamidesodium powder), mandelic acid (α -hydroxyphenylacetic solution 20% w/v) and lactic acid (DL-lactic acid powder) were obtained from Sigma Chemical Company (St Louis, MO, USA). Fresh solutions were prepared in double distilled water (except phenoxyethanol, which was dissolved in ethanol and further diluted in water to the desired concentration) before every experiment. Neutralizer was composed of 30 mL Tween 80 + 3 g lecithin + 5 g sodiumthiosulphate + 1 g L-histidine + 1 mL 0.25 M potassium phosphate buffer and distilled water ad 100 mL (Statens Serum Institut). In preliminary experiments the neutralizer was proved to terminate the action of all antiseptics in less than 1 min and did not affect the growth curve (data not shown).

Determination of MIC and MBC

MICs and MBCs were determined by a broth dilution method in microtitre plates (Nunc, Roskilde, Denmark) and in glass tubes. An overnight broth suspension was diluted to a final bacterial concentration of 10^5 – 10^6 cfu/mL with two-fold serial dilutions of each antiseptic together with an untreated control. The total volumes in microtitre wells were 200 μL , and in glass tubes 400 μL . After stationary incubation (*P. aeruginosa* was incubated on a rotary shaker, 100 rpm) at 35°C for 18–22 h in air the wells or tubes were examined for macroscopic growth. MIC was defined as the lowest concentration of antiseptic allowing no visible growth. Ten microlitres from each well or tube with no visible growth were mixed with 90 μL of neutralizer and, after mixing, 10 μL in duplicates were spotted on blood-agar plates. After incubation in air at 35°C for 18–22 h MBC was recorded as the lowest concentration of antiseptic that killed 99.9% of the original number of the pathogen.

Determination of lag of regrowth and bactericidal activity

These experiments were carried out as suspension tests at 35°C . Log-phase cultures of approximately 10^7 cfu/mL were mixed in glass tubes in a total volume of 2 mL mixed with the different antiseptics at the desired concentration or with a drug-free control. Two different treatment regimens were employed: one protocol used $1 \times \text{MBC}$ for 1 h. The second protocol, with a contact time of 2 min, used fixed suboptimum concentrations of each antiseptic (optimum concentrations sterilized the culture, impeding evaluation of a lag), which in preliminary experiments were adjusted to provide definable and reproducible killing effect. After static incubation (except for *P. aeruginosa*) at 35°C for 2 min or 1 h in air the action of antiseptics was terminated by a neutralization-dilution procedure. Aliquots of 100 μL were removed from each culture and diluted in 900 μL prewarmed (35°C) neutralizer. After mixing and incubation on the table for 2–3 min, 200 μL of each suspension was mixed with 1800 μL prewarmed MHB and incubated without shaking (except for *P. aeruginosa*) at 35°C . The resulting regrowth curve was constructed by viable counts at the time of drug-inactivation and at appropriate intervals thereafter (up to 8 h). Aliquots were removed from all cultures and diluted serially in saline and plated as 20 μL spots in duplicate on 5% blood-agar plates (undiluted suspensions were plated with 100 μL). After incubation for 18–24 h in air at 35°C , cfu were counted.

Duration of lag of regrowth was calculated by means of the equation $\text{lag (time)} = T - C$, where T is the time required for the cfu count in the test culture to increase one \log_{10} above the count immediately after the dilution procedure, and C is the time required for the cfu count in an untreated culture to increase by one \log_{10} above the count observed after drug removal. A significant lag of regrowth was defined as a lag of 0.5 h or more.

The bactericidal activity of each antiseptic was determined after 2 min or 1 h of drug exposure as part of each lag of regrowth experiment. The bactericidal rate was calculated from the \log_{10} count determined after drug exposure compared with the initial counts.

Statistics

The following statistical tests were used. For comparison of MIC or MBC determined in microtitre plate versus glass tube: three-way analysis of variance. For comparison of paired means: Wilcoxon matched-pair test. Spearman's test was used for correlation analysis. A level of $P < 0.05$ was considered significant.

Results

The MICs and MBCs determined are shown in Table I. The MICs were not influenced by the material of the test vessels

Antiseptics and lag of regrowth

Table I. Median (and range) of MIC and MBC determined in glass tubes and microtitre plates for nine bacterial species

Disinfectant	Median (range) of MIC and MBC (mg/L)			
	MIC in glass tubes	MBC in glass tubes	MIC in microtitre plates	MBC in microtitre plates
Chloramine T	794 (500–2000)	926 (500–2000)	857 (500–1000)	1000 (500–2000)
Chlorhexidine	5.0 (3.12–6.25)	5.8 (3.12–12.5)	3.7 (0.8–12.5)	6.8 (1.6–50)
Povidone-iodine	2361 (1250–2500)	2500 (2500)	2314 (1250–2500)	3402 (2500–5000)
Phenoxyethanol	5555 (6000–12 000)	9524 (3000–24 000)	6000 (3000–12 000)	19049 (6000–48 000)
Mandelic-lactic acid	729 (625–2500)	1157 (325–2500)	1575 (1250–2500)	2500 (1250–5000)

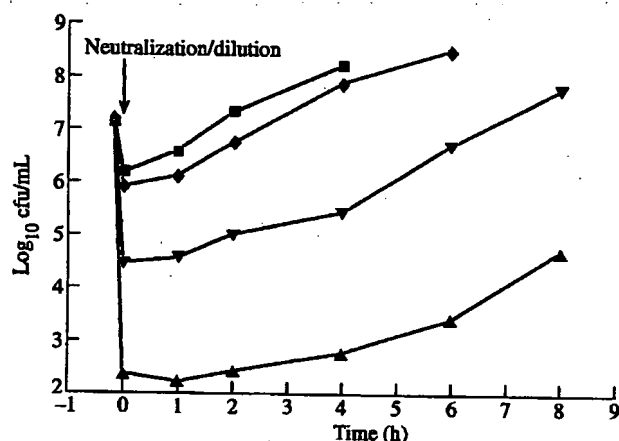


Figure. Regrowth kinetics of an *E. faecalis* strain exposed for 2 min to 0.2% chloramine T, 0.01% chlorhexidine or 1.2% phenoxyethanol. ■, Control; ◆, phenoxyethanol (lag = 0.3 h); ▲, chlorhexidine (lag = 2.1 h); ▼, chloramine T (lag = 4.2 h).

($P > 0.05$), whereas MBCs determined in microtitre wells were significantly higher than those determined in glass tubes ($P = 0.02$). All subsequent experiments were performed in glass tubes.

The bactericidal activity of the five disinfectants or antiseptics was highly dependent on the test concentration. Preliminary experiments using concentrations derived from Sticker *et al.*^{6,8} (i.e. chlorhexidine, 0.02%; povidone-iodine, 1%; phenoxyethanol, 2.4%; mandelic-lactic acid, 1%) produced a $>5 \log_{10}$ reduction after 1–2 min for all bacterial species. Thus to obtain a definable and reproducible killing effect following a 2 min contact time it was necessary to decrease the test concentrations to the following: chloramine T, 200 mg/L = 0.2% w/v; chlorhexidine, 10 mg/L = 0.01% v/v; povidone-iodine, 500 mg/L = 0.5% w/v;

phenoxyethanol, 1200 mg/L = 1.2%; mandelic-lactic acid, 500 mg/L = 0.5%. The killing rates obtained using the two treatment protocols are given in Table II. Only a few discrepancies could be demonstrated comparing the two regimens. The killing rates of chloramine T, chlorhexidine and povidone-iodine were comparable and significantly superior compared with those of phenoxyethanol and mandelic-lactic acid.

Regrowth curves from a typical experiment with *E. faecalis* can be seen in the Figure. The reproducibility of the method for determination of lag of regrowth was acceptable with an overall mean coefficient of variance of 22%. The lag of regrowth clearly depended on both the type of antiseptic and the bacterial species, and no single antiseptic produced a significant delay in regrowth on all bacterial species tested (Table III). Mandelic-lactic acid and chloramine T induced a significantly longer lag compared with the other agents, whereas phenoxyethanol clearly was the least active antiseptic in terms of lag of regrowth. No significant correlation between the delay in regrowth and result of the Gram's stain or killing rate could be demonstrated.

Discussion

MBCs of the tested antiseptics were significantly increased when determined in microtitre wells compared with those in glass tubes. This could be caused by bacteria adhering to the surface of the microtitre well leading to increased counts of survivors, which has been observed in MBC determinations of oxacillin against *S. aureus*.⁹ However, the significance of this observation is difficult to assess as the contact time when testing antiseptics is short (usually a few minutes) compared with MBC testing (overnight incubation).

Data presented in this study confirmed that antiseptics can also induce suppression of regrowth—an equivalence

Table II. Bactericidal activity of five disinfectants^a on nine bacterial species after exposure for 2 min at the indicated concentrations and for 1 h at 1 × MIC

Species	Log ₁₀ decreases in viability counts (mean values of 2–4 separate experiments)									
	chloramine T		chlorhexidine		povidone-iodine		phenoxyethanol		mandelic-lactic acid	
	2 min	1 h	2 min	1 h	2 min	1 h	2 min	1 h	2 min	1 h
<i>S. epidermidis</i>	2.7	2.7	3.7	2.2	3.9	2.7	0.9	0	2.0	0.5
<i>S. aureus</i>	3.1	4.0	2.7	1.6	2.8	2.9	0.2	0.1	0.8	1.0
<i>E. faecalis</i>	4.6	4.5	1.6	1.7	0.8	1.5	0.2	0.3	0.4	0.9
<i>E. coli</i>	1.7	1.9	2.5	3.0	2.3	4.3	2.1	0.6	2.0	2.0
<i>K. pneumoniae</i>	1.4	1.0	2.4	3.3	3.4	1.9	1.3	0.2	1.2	2.7
<i>S. marcescens</i>	1.9	1.4	1.6	2.9	2.5	1.6	1.0	−0.1	1.3	2.5
<i>P. mirabilis</i>	3.2	4.2	1.6	3.6	3.4	2.3	0	0	1.1	1.7
<i>E. cloacae</i>	2.7	2.7	2.6	2.0	1.8	3.0	0.8	−0.1	1.8	0.8
<i>P. aeruginosa</i>	1.2	0.8	2.5	1.8	2.2	2.4	0.9	0.8	3.5	1.3
Mean for all strains	2.7	2.7	2.5	2.2	2.5	2.4	0.9	0.1	1.3	1.3

^aChloramine T, 0.2%; chlorhexidine, 0.01%; povidone-iodine, 0.5%; phenoxyethanol, 1.2%; mandelic-lactic acid, 0.5%.

Table III. Duration of lag of regrowth of five disinfectants^a on nine bacterial species after exposure for 2 min at the indicated concentrations and for 1 h at 1 × MIC

Species	Duration of lag of regrowth (h) (mean values of 2–3 separate experiments)									
	chloramine T		chlorhexidine		povidone-iodine		phenoxyethanol		mandelic-lactic acid	
	2 min	1 h	2 min	1 h	2 min	1 h	2 min	1 h	2 min	1 h
<i>S. epidermidis</i>	3.2	2.0	3.5	2.0	1.6	0.9	1.4	0.5	2.7	3.5
<i>S. aureus</i>	2.3	1.3	2.9	2.3	2.1	0.9	2.2	0.9	1.5	2.0
<i>E. faecalis</i>	4.0	4.8	2.1	1.6	1.7	1.2	0.4	0.1	0.5	0.6
<i>E. coli</i>	1.7	2.0	1.8	0.9	0.5	0.5	0.1	0.1	2.1	1.2
<i>K. pneumoniae</i>	2.2	1.4	1.6	0	1.3	0.9	0.2	0.2	2.4	2.9
<i>S. marcescens</i>	2.9	2.7	2.6	0.9	2.8	0.4	2.1	0.2	4.4	2.3
<i>P. mirabilis</i>	0.8	2.6	1.3	1.0	1.0	1.6	0.1	0.3	3.6	1.3
<i>E. cloacae</i>	3.6	4.6	1.7	0.1	2.1	1.3	−0.4	−0.1	2.1	0.3
<i>P. aeruginosa</i>	−0.1	0.1	3.5	4.3	1.6	0.9	2.2	5.1	5.7	4.7
Mean for all strains	2.3	2.0	2.1	1.0	1.6	0.9	0.4	0.2	2.4	2.0

^aChloramine T, 0.2%; chlorhexidine, 0.01%; povidone-iodine, 0.5%; phenoxyethanol, 1.2%; mandelic-lactic acid, 0.5%.

to PAE after induction with an antibiotic. The reproducibility of the method used for assessment of this lag phase was reliable although the overall coefficient of variance was higher (22%) compared with experiments with antibiotics (12%).¹⁰ The five antiseptics tested in this study displayed different potencies with respect to both killing rate and lag of bacterial regrowth. However, it is important to note that the antiseptics could not be evaluated at the optimum concentrations used clinically. Consequently, the data cannot be compared in absolute terms although the results obtained with equipotent concentrations revealed comparable results. Comparative data in the literature on lag of regrowth induced by antiseptics are limited^{2–4} but some studies exist on the extent of injury

induced by various antiseptics, manifested by the inability of damaged bacteria to form colonies on various selective media.^{11–13} In one paper the lag times of injured bacteria were used to assess differences in repair time following short-term exposure to various stimuli.¹⁴ Those authors observed that lag times of injured *Salmonella typhimurium* produced by different types of stress were a convenient approach to evaluate differences in repair times versus lag times.¹⁴ These observations suggest that the time to repair sublethal injury is equivalent to the lag of regrowth. Exposure of *Bacillus megaterium* to 12 µM chlorhexidine for 5 min has been shown to require 1–2 h for recovery from this injury.¹⁵ Treatment of *E. coli* with a sublethal concentration of chlorine induced a lag of around 2 h,² whereas no

Antiseptics and lag of regrowth

lag was seen using *Pseudomonas fluorescens* as the target organism.³ A similar species-dependent lag of regrowth was observed in the present study. Acidification of *E. coli* has also been shown to cause sublethal injury.¹⁶⁻¹⁸ In one study, complete repair was observed after 1-2 h following induction with 0.3 M sodium acetate at pH 4.2 for 60 min,¹⁸ which is comparable to the 1.2-2.1 h lag found in this study on *E. coli* induced with mandelic-lactic acid.

Demonstration of a short lag of regrowth indicates that the injury involved is repaired rapidly. It is possible to characterize the damage to the cytoplasmic membrane either as a chemical injury (implying that the membrane is destroyed, which requires resynthesis of macromolecules for repair—a slow process) or a physical injury (alteration of configurations of membrane components, which merely requires reorganization for repair—a rapid process).¹⁹ Phenoxyethanol (an uncoupling agent)^{20,21} and chlorhexidine (an inhibitor of membrane-bound ATPase)^{15,21,22} both act predominantly at the cytoplasmic membrane, presumably by induction of physical injury to the membrane.¹⁹ The short lag of regrowth observed in this study with phenoxyethanol supports this mechanism of action. However, it does not explain the long lag demonstrated in *P. aeruginosa* or why we did not observe a consistency between the spectrum and extent of lag of each antiseptic on the various microorganisms.

Clinical studies testing disinfectants and antiseptics have also observed a sustained antibacterial effect. However, it is important to recognize that such studies do not include a neutralization step. Consequently, demonstration of a sustained antibacterial effect in clinical studies is probably a combination of lag of regrowth and residual activity of disinfectants and antiseptics on site, as has been confirmed on the skin²³ and in the urinary bladder.²⁴ One clinical trial examining the effect of intermittent bladder irrigation (once or twice daily) on bacterial counts with an indwelling catheter observed a lag of regrowth with 0.02% chlorhexidine whereas no lag was observed after washouts with saline or 0.25% acetic acid.²⁵ A number of studies on the efficacy on skin disinfection have shown that solutions containing chlorhexidine, and, to a lesser extent, isopropanol or hexachlorophane, demonstrate a sustained antibacterial effect on the skin flora, in contrast to povidone-iodine or alcoholic solutions.^{26,27} Various antiseptic solutions for mouthwash application in the reduction of oral microbial flora have also been studied with respect to a sustained antibacterial effect. Formulations containing chlorhexidine have in general been shown to demonstrate the most extended antibacterial effect²⁸⁻³⁰ in contrast to those containing ethanol as the major component.^{28,29}

The aim of any antibacterial regimen is to maximize the positive effects (i.e. killing) during the period when active drug levels are present and to minimize the negative effects (i.e. regrowth) during the inactive period when subinhibitory levels of drug are present. Thus measurements of both the bactericidal activity and the lag time could

be useful in screening the efficacy of antiseptics and contribute to the choice of antiseptic. Moreover, such information could guide in determining the optimum interval between repeated applications of antiseptics. Further data are clearly needed for a final assessment of the significance of lag of regrowth of antiseptics.

It is concluded that, although in-vitro determination of bactericidal rate and lag of regrowth does not replace standard evaluation of antiseptics, it provides a cheap and easy way of screening antiseptics.

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Received 10 September 1996; returned 21 November 1996; revised 23 December 1996; accepted 10 March 1997

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Chloramine-T

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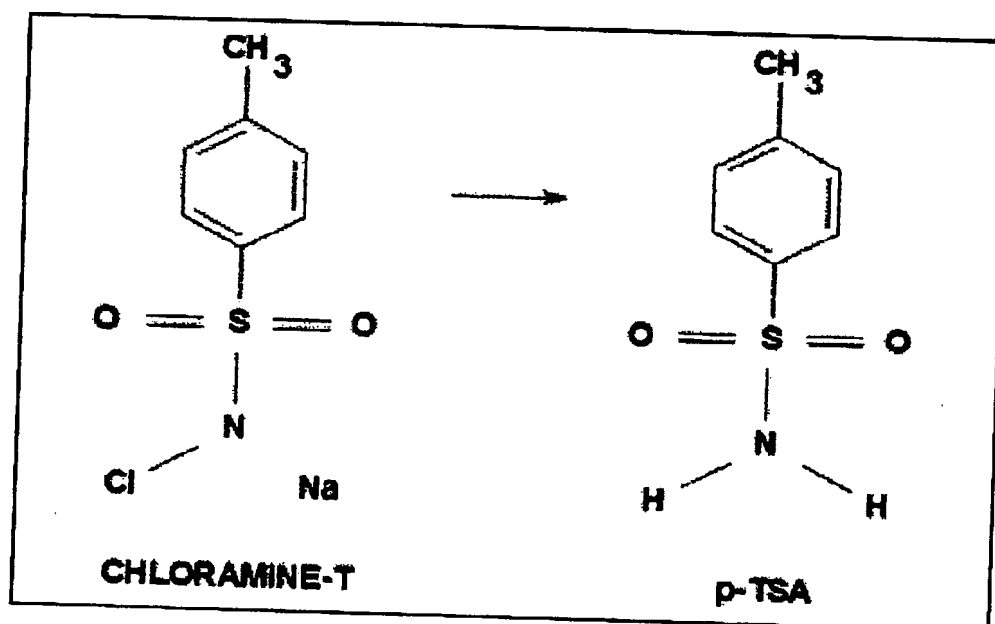
Public hatcheries in the United States, including facilities culturing threatened and endangered fish, are severely restricted by the lack of legally approved medicinal drugs to treat diseased fish. Chloramine-T is a drug that has great potential to control and reduce mortalities associated with a number of external fish diseases.

Chloramine-T was identified by state and federal hatcheries as an urgently needed drug that will be used to reduce disease-related mortalities and improve production efficiency and product quality at public hatcheries. Studies required by the Food and Drug Administration were conducted to allow the approved use of



chloramine-T to treat bacterial diseases on cold, cool, and warm water fish cultured in public hatcheries.

Measuring chloramine-T concentrations in exposure water.



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Page Last Modified: March 31, 2006



**Chloramine-T
[127-65-1]
and
Metabolite *p*-Toluenesulfonamide
[70-55-3]**

Review of Toxicological Literature

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February 2002

**Chloramine-T
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Review of Toxicological Literature

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Toxicological Summary for Chloramine-T [127-65-1] and *p*-Toluenesulfonamide [70-53-3]

Feb/02

Executive Summary

Nomination

Chloramine-T was nominated by a small commercial organization for toxicology studies based on its current status as an Investigational New Animal Drug (INAD) for controlling proliferative gill disease and bacterial gill disease in aquaculture and the need for additional toxicology studies to support its safe use. The metabolite *p*-Toluenesulfonamide (*p*-TSA) is of importance as the primary residue of chloramine-T in chloramine-T treated fish intended for human consumption.

Non-toxicological Information

Chloramine-T, as an anti-microbial agent, has had widespread use in a broad range of practices, including medical, dental, veterinary, food processing, and agricultural. As a disinfectant, it is used to disinfect surfaces and instruments. Chloramine-T has a low degree of cytotoxicity and has been used in direct contact with tissues. As such, it is used in the treatment of burns, in whirlpools for the treatment of wounds, and as an oral mouthwash. In agricultural practices, chloramine-T has been approved as a broad-spectrum biocide for foot-and-mouth disease, swine vesicular disease, diseases of poultry, and tuberculosis in the United Kingdom, and is used in numerous branches of industry such as intensive farming, slaughterhouses, and kitchens. Within the United States, use of chloramine-T is more restricted. EPA registration for eating establishment utensils, barbershop instruments and as an herbicide was withdrawn between 1983 and 1987.

Currently, there is interest in obtaining approval from the Food and Drug Administration (FDA) for the use of chloramine-T in aquaculture. In 1994 the International Association of Fish and Wildlife Agencies (IAFWA) agreed to support research on a priority list of chemicals, including chloramine-T, identified as being important to various state fisheries. The list included chemicals there were needed but not currently labeled for aquaculture use. Data regarding target animal safety, efficacy, analytical methods, residue analysis, and metabolism studies, and environmental assessment were either collected or generated to fill data gaps for the investigational new animal drug (INAD)/new animal drug application (NADA) submission. In addition, toxicology data on *p*-TSA, the major metabolite of chloramine-T, was needed for the development of tolerances. In March 2001, concern over the potential carcinogenicity of *p*-TSA was voiced. Although work continues to gain FDA approval for chloramine-T, only laboratory studies or studies in which fish will not be released or slaughtered for food will be approved.

Chloramine-T is used in several other industries. It is used to bleach products (textiles and in the conservation of books), to dye textiles, as the starting material for other compounds, and as a laboratory reagent.

p-TSA is used as an intermediate for pesticides and drugs and is used as an additive to outdoor paints in Sweden. Mixtures of *o*- and *p*-TSA are used as reactive plasticizers in hot-melt adhesives to improve flow properties of thermosetting resins. The mixture also adds flexibility to coatings based on some resins. The TSA mixture is used as a carrier in fluorescent pigments. Both *o*- and *p*-TSA were common, and quantitatively important, contaminants of saccharin produced by the Remsen and Fahlberg process, and therefore have been studied for their ability to cause mutations.

p-TSA is used in the formulation of toluenesulfonamide/formaldehyde resin (TSFR), which is used in fingernail polishes and enamels at concentrations up to 10%.

The United States aggregated production volumes for chloramine-T were reported to be between 10,000 and 500,000 lbs. in 1998. Total aggregated production of *p*-TSA was reported between one and ten million lbs. for the same year.

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Based on the screening information data set (SIDS) initial assessment report (SIAR) developed by the OECD, *p*-TSA was considered to be "presently of low concern." The OECD SIAR concluded that "although 4-methylbenzenesulfonamide [*p*-TSA] is persistent and toxicological tests showed moderate toxicity, no further testing is needed at present considering its use pattern and exposure levels", and further recommended that "if this chemical is used largely in consumer products in the future, long-term repeated dose (e.g. 90 days) toxicity test may be needed, because histopathological changes of urinary bladder and thymus were observed in combined repeat/repro. toxicity test."

Human Data

Exposures to chloramine-T, *p*-TSA, and TSFR are likely to occur whenever they are used in the above-mentioned practices. Chloramine-T is typically purchased in solid form and is then made into solutions. Either the solid or the liquid forms may produce adverse reactions. There is a potential for oral exposures to chloramine-T based on residue carry-over from food industries where it may be used to disinfect surfaces. Occupational exposures to *p*-TSA are likely in the production of other chemicals derived from it or through its addition to paints. Restrictions have been made on the amount of total TSAs allowed as contaminants of saccharin. Exposures to TSFR are usually associated with the use of nail polishes.

Chloramine-T is considered harmful if swallowed, inhaled, or absorbed through the skin or eyes. Symptoms include burning sensation, coughing, wheezing, laryngitis, shortness of breath, sore throat, bronchitis, pneumonia, and possible pulmonary edema. Chloramine-T dust is irritating to the eyes. Skin contact with chloramine-T produces redness, itching, and pain, with a potential for the development of an allergic skin reaction. A probable oral lethal dose of 0.5 to 5 g/kg (1.8 to 18 mmol/kg) for a 70 kg individual has been suggested.

Exposures to chloramine-T have the potential to result in hypersensitivity or occupational asthmatic reactions. Case reports discuss urticaria and respiratory problems associated with occupational exposures. This has been supported by positive results for chloramine-T in the guinea pig maximization test, local lymph node assay, and Buehler occluded patch test.

TSFR has also been associated with hypersensitivity reactions, typically in areas that come in frequent contact with fingernails. One case report described the development of onycholysis of the fingernails that resolved once the use of nail products was discontinued.

Animal Studies***Chemical Disposition, Metabolism, and Toxicokinetics***

In fish studies, chloramine was poorly absorbed from water, and that which was absorbed was rapidly metabolized to the residue marker, *p*-TSA. A second, as of yet unidentified, metabolite may also exist.

In the rat, chloramine-T is rapidly distributed throughout the body. Plasma clearance is relatively rapid, with elimination primarily through the urine. *p*-TSA is rapidly eliminated from rats, also. The primary metabolite found in the urine is 4-sulphamoylbenzoic acid. The data suggest that the methyl group of *p*-TSA is oxidized to primarily the benzoic acid derivative.

Chloramine-T, used as an udder wash, was poorly absorbed percutaneously, and rapidly metabolized to *p*-TSA.

Acute Toxicity

Acute toxicity values for chloramine-T reported in mice were 1100 mg/kg (3.9 mmol/kg) *per os* (p.o.) (LD₅₀) and 300 mg/kg (1.07 mmol/kg) intraperitoneally (i.p.) (LD₅₀). For rats, the values were 0.275 mg/L (0.976 µM) via inhalation (LC₅₀) and 935 mg/kg (3.32 mmol/kg) p.o. (LD₅₀). In Guinea pigs, the

Toxicological Summary for Chloramine-T [127-65-1] and *p*-Toluenesulfonamide [70-55-3]

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LD_{Lo} was reported as 900 mg/kg (3.20 mmol) subcutaneously (s.c.), and in rabbits, the dermal LD₅₀ was greater than 2000 mg/kg (7.1 mmol/kg), while the intravenous LD_{Lo} was 25 mg/kg (89 µmol/kg).

The LD₅₀s for *p*-TSA in mice were 400 mg/L (2.34 mM) p.o. and 250 mg/kg (1.46 mmol/kg) i.p. In rats, the oral LD₅₀ was reported to be greater than 2000 mg/L (11.68 mM). In guinea pigs, the s.c. LD_{Lo} was 2 g/kg (0.01 mol/kg).

Chloramine-T is irritating to the skin, eyes, and gastrointestinal tract. In acute LD₅₀ studies, gastric inflammation, apathy, gastric bleeding and intestinal hemorrhage was observed in the animals that died. *In vivo* and *in vitro* animal studies using chloramine-T indicate an ability to affect elastase inhibitory capacity (EIC) negatively. An age-dependent toxicity was observed in rabbits, with young rabbits more susceptible than adults.

TSFR was found to be relatively non-toxic in rats or rabbits when tested acutely through oral, ocular, or dermal exposures.

Short-Term and Subchronic Toxicity

In the rat, higher doses of chloramine-T over 28 to 90 days resulted in reduced weight gains and increased relative kidney and liver weights. Leukocyte counts were slightly elevated and livers were discolored in the 28-day study. Female rats demonstrated an increased severity and frequency of calcareous deposits in the kidneys. Similar results were observed in dogs. In addition, hematological and clinical chemistry changes were observed in some treatment groups. Histopathological examination showed a thickening of the urinary bladder epithelium. TSA (mixture of *ortho*- and *para*- isomers in the diet) resulted in a slight reduction in food consumption and weight gain in dogs. No significant treatment-related lesions were observed in dogs fed TSFR in their diets for 90 days.

Chronic Toxicity

Chronic exposure of dogs to chloramine-T resulted in a persistent mild or moderate anemia and a reduction in EIC in both serum and pulmonary lavage fluids.

Reproductive and Teratological Effects

Maternal toxicity expressed as decreased weight gain was observed in several teratology studies for *p*-TSA. One study reported difficult labors in two of the rats in the 750 mg/kg/d [4.38 mmol/kg/d] dose group along with 100% mortality of offspring by day three of lactation. Increased resorption rates and postimplantation losses were observed at doses as low as 250 mg/kg/d [1.46 mmol/kg/d]. Fetal body weights were reduced at 50 mg/kg/d [0.29 mmol/kg/d]. Increased unossified sternebrae were observed at 500 mg/kg/d [2.92 mmol/kg/d]. A no observed adverse effect level and no observed effect level were estimated as 300 and 50 mg/kg/d [1.75 and 0.29 mmol/kg/d], respectively, by two different studies.

No differences in mating performance or fertility were observed in reproductive studies of *p*-TSA, nor were any differences in reproductive parameters found between treatment groups. Neonatal survival and body weights were significantly decreased at the 750 mg/kg/d treatment levels.

Genotoxicity

The genotoxic potential of chloramine-T has been studied in a variety of systems, with mostly negative results. A statistically significant dose-dependent increase in sister chromatid exchanges was observed in one study. The effect was reduced, but not abolished, with the addition of methionine.

p-TSA has undergone more extensive genotoxicity testing. A single study reported a weak mutagenic effect in a modified *Salmonella*/microsome assay. Positive results were also noted when tested in the

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Drosophila assay. *p*-TSA was negative in Chinese hamster lung cells, Chinese hamster ovary cells, R5a cells, and in the micronucleus assay.

Other Data

Chloramine-T has the ability to bind to enzymes and alter their characteristics. Plasma-kallikrein treated with chloramine-T was not cleared from the liver. The spectral characteristic of serum albumin treated with chloramine-T was altered. Chloramine-T impacts a variety of serine protease inhibitors, decreasing their effectiveness. Methionine and tyrosine residues represent susceptible sites.

Several investigators have examined the potential for using chloramine-T to develop an animal model for chronic human α_1 -proteinas inhibitor deficiency, mostly without success, as the changes induced by chloramine-T are transient in nature.

In wound healing studies, chloramine-T was shown to be less irritating than hypochlorite solutions, though it was also less effective in cleaning the wound. Chloramine-T also significantly delayed the production of collagen and prolonged the acute inflammatory responses relative to saline.

Chloramine-T has been used as a surrogate chemical to study voltage-gated sodium channels and the effects of free radicals and activated oxygen compounds associated with brain ischemia and head trauma. One case study was identified that investigated the possible potentiation of formate-induced blindness by chloramine ingestion.

No studies were identified for synergistic/antagonistic effects, carcinogenicity, initiation/promotion, anticarcinogenicity, cogenotoxicity, antigenotoxicity, or immunotoxicity of chloramine or *p*-TSA.

Structure-Activity Relationships

o-Toluenesulfonamide (*o*-TSA) is the major contaminant in saccharin produced by the Remsen and Fahlberg process. Numerous studies have been conducted examining the toxicities of *o*-TSA. At low oral doses, humans excreted *o*-TSA more slowly than rats.

LD₅₀'s for *o*-TSA in rats ranged from 2 g/kg to 4,870 mg/kg [11.68 to 28.44 mmol/kg]. Applied to the eyes of rabbits, *o*-TSA was rated as a moderate irritant at the 24-hour endpoint. In long-term assays (two to six generations) low numbers of animals developed benign bladder tumors, including the control groups. Dose-related incidences in bladder calculi were found in the offspring of rats dosed throughout gestation and lactation. *o*-TSA predisposes neonatal animals to urolithiasis and/or bladder lesions.

When tested for the ability to cause morphological changes in the eye lens, *o*-TSA was almost inactive.

Results in mutagenicity studies of *o*-TSA have been somewhat equivocal. Mutation rates were doubled in TA98 at very high doses in the presence of an S9 fraction, but only on a special medium. Several *Drosophila* studies reported either negative or weakly positive results. One study demonstrated statistically significant doubling frequencies after three days' feeding of *o*-TSA. *o*-TSA was negative in mammalian systems.

In carcinogenicity studies, lymphosarcomas were observed in all dose groups exposed to *o*-TSA. Papillomas of the bladder were found in both low-dose and high-dose groups, with one carcinoma of the bladder observed. The incidence of malignant tumors was no different in the treated groups than in the control groups. In a separate study, no bladder tumors were observed though a mild diffuse urothelial hyperplasia was found in one rat.

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1.0 Basis for Nomination

Chloramine-T was nominated by a small commercial organization for toxicology studies based on its current status as an Investigational New Animal Drug (INAD) for controlling proliferative gill disease (PGD) and bacterial gill disease (BGD) in aquaculture and the need for additional toxicology studies to support its safe use. The metabolite *p*-Toluenesulfonamide (*p*-TSA) is of importance as the primary residue of chloramine-T in chloramine-T treated fish intended for human consumption.

2.0 Introduction

Chloramine-T has been used as a disinfectant since the early 1900s in a wide variety of industries that range from hospital to agricultural use. It is effective against a large number of bacteria and viruses without inducing drug resistance. The aquaculture industry has become very interested in developing Chloramine-T for use as a therapeutic against PGD and BGD. Investigational new animal drug applications (INADs) have been submitted to the U.S. Food and Drug Administration (FDA) to support the approval of chloramine-T for this use in the United States. In March 2001, the FDA announced a concern that the residue marker for chloramine-T, *p*-toluenesulfonamide, might be a carcinogen, and that no slaughter authorizations for fish treated with chloramine-T would be approved after the current INADs expired. The focus of this report is chloramine-T and its metabolite, *p*-toluenesulfonamide (*p*-TSA).

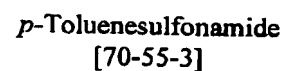
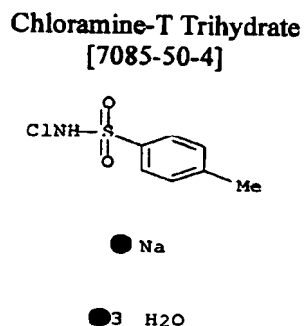
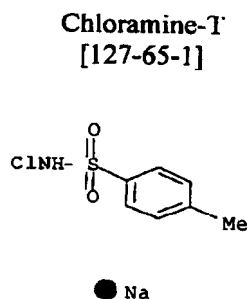
Chloramine-T should not be confused with the generic chloramine. In the municipal water treatment industry, chloramine refers to a combination of chlorine and ammonia used by some communities as an alternate to chlorination. Chloramination of drinking water is less effective than chlorine and may cause adverse health effects by both damaging and interfering with the repair of red blood cells. Where water is chloraminated, dialysis centers have had to treat their water with a combination of reverse osmosis and charcoal filtration systems to prevent anemia in hemodialysis patient (Tibbetts, 1995).

Chloramines may also be created endogenously through inflammatory processes. Myeloperoxidase, secreted by stimulated monocytes and neutrophilic polymorphonuclear leukocytes, catalyzes the oxidation of chloride by H_2O_2 to form hypochlorous acid (HOCl). Hypochlorous acid in turn reacts with ammonia and amines to form chloramines (*N*-Cl) (Thomas et al., 1987). Much of the scientific literature regarding chloramines uses chloramine-T as a surrogate for these endogenously formed chloramines to study the mechanisms by which they exert their activities.

The term chloramine-T has been used without distinction between the anhydrous form [CAS RN 127-65-1] and the trihydrate [7085-50-4] forms. As most of the Material Safety Data Sheets (MSDS) cited the molecular weight of the trihydrate while listing the anhydrous CAS RN, the assumption was made that the manufacturers were supplying the trihydrate. Therefore, the molecular weight of the trihydrate was used in converting doses in the toxicology sections.

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2.1 Chemical Identification and Analysis

Chloramine-T ([C₇H₇ClNNaO₂S]; mol. wt. = 227.65) is also called¹:

Benzenesulfonamide, *N*-chloro-4-methyl, Sodium salt (CA Index Name)
p-Toluenesulfonamide, *N*-chloro-, sodium salt
 Actamid
 Acti-chlore
 Aktivin
 Anexol
 Aseptoclean
 Berkendyl
 Chloralone
 Chloramine-T
 Chlorasan
 Chloraseptine
 Chlorazan
 Chlorazene
 Chlorazone
 Chlorozone
 Chlorseptol
 Cloramine T
 Clorina
 Clorosan
 Desinfect
 Euclorina
 Gansil
 Gyneclorina

Halamid
 Helogen
 Kloramin
 Kloramine-T
 Mannolite
 Mianine
 Monochloramine T
 Multichlor
N-Chloro-4-methylbenzylsulfonamide sodium salt
N-Chloro-*p*-toluenesulfonamide sodium
N-Chloro-*p*-toluenesulfonamide sodium salt
N-Chlorotoluenesulfonamide sodium salt
 Sodium chloramine T
 Sodium *N*-chloro-4-methylbenzenesulfonamide
 Sodium *N*-chloro-*p*-toluenesulfonamide
 Sodium *p*-toluenesulfochloramide
 Sodium *p*-toluenesulfonchloramide
 Sodium *p*-toluenesulfonylchloramide
 Sodium tosylchloramide
 Tampules
 Tochlorine
 Tolamine
 Tosylchloramide sodium

¹ Budavari, 1996

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Chloramine-T trihydrate ($[\text{C}_7\text{H}_7\text{ClSO}_2\text{N NaCl} (3\text{H}_2\text{O})]$; mol. wt. = 281.69) is also called²:

Benzenesulfonamide, *N*-chloro-4-methyl-, sodium salt, trihydrate
Sodium, (*N*-chloro-*p*-toluenesulfonamido)-, trihydrate
Tosylchlora

p-Toluenesulfonamide ($[\text{C}_7\text{H}_9\text{NO}_2\text{S}]$; mol. wt. = 171.23) (*p*-TSA) is also called:

Benzenesulfonamide, 4-methyl- (CA Index Name)	<i>p</i> -Tolylsulfonamide
4-Methylbenzenesulfonamide	<i>p</i> -Tosylamide
4-Methylphenylsulfonamide	Plasticizer 15
PTSA	Toluene-4-sulfonamide
<i>p</i> -Toluenesulfamide	Toluene- <i>p</i> -sulfonamide
<i>para</i> -Toluenesulfamine	4-Toluenesulfonic acid, amide
<i>para</i> -Toluenesulfonamide	Tolylsulfonamide
4-Toluenesulfanamide	<i>p</i> -Tolylsulfonamide
4-Tolylsulfonamide	Tosylamide
<i>p</i> -Methylbenzenesulfonamide	<i>p</i> -Tosylamide
4-MBSA	Uniplex 173

Synthesis and Analysis

Chloramine-T is synthesized from methylbenzene and four volumes of chlorosulfonic acid, which are allowed to react together at less than -5°C . This reaction results in equal amounts of *ortho*- and *para*-toluenesulfonyl chloride. Pouring the mixture over ice separates the isomers. The *p*-toluenesulfonyl chloride crystallizes out of the mixture while the *o*-toluenesulfonyl chloride remains in solution. *p*-Toluenesulfonyl chloride is treated with ammonia to yield *p*-Toluenesulfonamide, followed by sodium hypochlorite treatment to form chloramine-T (Omikron-Online, 2000).

Many analytical methods have been developed to detect residues of both chloramine-T and its major metabolite (*p*-TSA) in a variety of foods (milk, ice cream, whole eggs, mechanically deboned poultry meat and croquettes (Beljaars et al., 1994; Steverink and Scholtyssek, 1977; Appel et al., 1988b). Most recently, isocratic reverse-phase liquid chromatography (absorbance detection set at 226 nm) has been used to measure chloramine-T's drug marker residue (*p*-TSA) in edible tissues of fish. Meinertz et al. (1999) reported mean recoveries of *p*-TSA ranging from 77 to 93.17% (method quantitation limits: 13 to 18 ng/g [0.076 to 0.11 nmol/g]; method detection limits: 3.8 to 5.2 ng/g [0.022 to 0.030 nmol/g]).

To detect chloramine-T or *p*-TSA in water, a reverse-phase liquid chromatographic method with ion suppression, using 0.01M phosphate buffer at pH 3, may be used (Dawson and Davis, 1990 abstr., 1997 abstr.). The mobile phase involves phosphate buffer-acetonitrile at one mL/min. Both chemicals can be detected with an UV spectrophotometer at 229 nm. Mean recoveries were about 95 or 96% for water samples fortified with 0.005 mg *p*-TSA/L [0.030 $\mu\text{mol/L}$] or

² ThermoNESLAB MSDS, 2000

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0.03 mg chloramine-T/L [0.11 $\mu\text{mol/L}$], respectively. Limits of detection were 0.001 [0.006 $\mu\text{mol/L}$] and 0.01 mg/L [0.036 $\mu\text{mol/L}$], respectively.

Van Gils et al. (1975) describe a method for the quantitative determination of traces of chloramine-T in dairy products. After removing proteins, the samples are extracted with ether. The solvent is removed and the residue oxidized with an alkaline potassium permanganate solution. This is extracted again with ether and the residue remaining after removal of solvent is subjected to reduction with Raney Nichol catalyst in a sodium hydroxide solution. The sulfonamide group is removed, leaving the benzoic acid, which is then analyzed by gas chromatography. Steverink and Scholtyssek (1977), describe a gas chromatographic determination of chloramine-T used in mechanically deboned chicken that is sensitive in the part per million (ppm) range with an average recovery of 80%.

Other methods used are specific for *p*- and/or *o*-TSA. The toluenesulfonamides are major impurities found in saccharine, one of the original non-nutritive sweeteners. Stavric et al. (1976) analyzed 13 saccharine samples used for cancer bioassay studies in a number of laboratories. The saccharine samples were dissolved in water and extracted with chloroform: methanol mixture (proportions not provided). The extract was concentrated and impurities were separated, underivatized, by gas liquid chromatography. Eleven major, well-separated peaks were collected, analyzed by mass spectroscopy, and compared with known standards. Quantitatively, *o*- and *p*-TSA were the most important impurities isolated and identified from saccharine.

Beginning around 1980, high performance liquid chromatography (HPLC) was used to isolate and quantify *p*-TSA. Duin and Nuijens (1981) analyzed dairy samples for *p*-TSA, first extracting the samples with acetone. The acetone extract was centrifuged and placed in a freezer for 30 minutes. The filtrate was analyzed by HPLC. In spiked ice cream samples, Duin and Nuijens achieved, on average, 92.8% recoveries of *p*-TSA. Mooser (1984), using reverse phase HPLC, reported detection limits for *o*- and *p*-TSA of eight and sixteen ng, respectively. The mobile phase was water and tetrahydrofuran. Recovery and linearity were tested in the five to one hundred mg/kg range.

Beljaars et al. (1993) combined dialysis with chromatography to measure *p*-TSA in ice cream. Samples were extracted with water and then dialyzed in a continuous flow system. Dialysates (500 μL) were injected onto a reverse-phase octadecylsilane bonded-phase (C-18) column and exposed to a methanol-water (25 + 75, v/v) mobile phase. Quantification was through a fluorescence detector (230 nm excitation, 295 nm emission). Mean recoveries using this method were 76 to 79%, with a range of 63 to 101%. This method was adopted (first action) by the Association of Official Analytical Chemists (AOAC) International in 1994 (Beljaars et al., 1994).

A method for the detection of *p*-TSA in baby food was described by Abete et al. (1996). As in the method of Beljaars, the samples were extracted with water. However, in this method, concentration of the sample was achieved through an Extrelut Column, followed by purification on a SPE C-18 cartridge. Samples were analyzed by a gas chromatography equipped with a flame ionization detector. Mean recoveries of *p*-TSA using this method were 85%.

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2.2 Physical-Chemical Properties Chloramine-T and *p*-Toluenesulfonamide

Property	Information	Reference(s)
<i>Chloramine-T</i>		
Physical State	White or slightly yellow crystals, crystalline powder, or prisms (trihydrate)	HSDB (2001b)
Odor	Weak chlorine odor (trihydrate)	HSDB (2001b)
Boiling Point (°C)	not applicable	Akzo Nobel (1998)
Melting Point (°C)	167 to 169; decomposes ca 174 (trihydrate)	H&S Chemical Co. Inc. (2000); (Akzo Nobel (1998); Physchem (1999)
Flash Point (°C)	192 (trihydrate)	Physchem (1999)
Density (kg/m ³)	1430	Akzo Nobel (1998)
Solubility (g/L) in:		
Water	150	Akzo Nobel (1998)
Benzene, chloroform, or ether	practically insoluble (trihydrate)	HSDB (2001b)
Specific Gravity	1.43 (trihydrate form)	Physchem (1999)
<i>p</i>-Toluenesulfonamide (<i>p</i>-TSA)		
Physical State	Solid (white leaflets) Solid (monoclinic plates) (dihydrate form)	HSDB (2001a)
Boiling Point (°C):		
at 1.33 kPa	214	HSDB (2001a)
at 10 mm Hg	221	OECD (1994)
Melting Point (°C)	137.5 138.5	OECD (1994) HSDB (2001a)
Flash Point (°C)	202	OECD (1994)
Solubility:		HSDB (2001a)
In water (g/L at 25 °C)	3.16	
In alcohol	Soluble	
Octanol-water partition coefficient (log P _{ow} , 25 °C)	0.82	HSDB (2001a)

Chloramine-T trihydrate is a stable material, but is incompatible with oxidizing agents (Physchem, 1999). Chloramine-T trihydrate may decompose violently if heated above 130°C, and may decompose on exposure to air. On combustion, chloramine-T trihydrate forms toxic and irritating gases (e.g., hydrogen chloride, and nitrogen, sulfur, and carbon oxides) (ILO, 1997).

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Chloramine-T has usually been classified as a slow hypochlorite-releasing agent (Axcentive, undated; Mavlab, undated). However, chloramine-T has several properties that do not fit the typical pattern of slow releasing agents, including stability in aqueous solutions, moderate pH dependency for biocidal efficacy, influence of organic matter on biocidal efficacy, minor skin irritation, and low chlorinating ability. Chloramine-T does not form chlorine or hypochlorous acids; therefore production of chlorinated organic compounds (AOX) is irrelevant (Axcentive, undated).

p-TSA is a non-volatile material, which is stable in neutral, acidic, or alkaline solutions (OECD, 1994).

2.3 Commercial Availability

Common vendors of chloramine-T are listed in Table 1. For the most part, chloramine-T is sold as a soluble concentrate (purity not provided). One source recommended a final concentration of 1% solution.

Chloramine-T trihydrate is available from many of the major chemical supply houses such as Fisher Scientific (Fairlawn, NJ [ThermoNESLAB MSDS, 2000] and Mallinckrodt Baker (Phillipburg, N.J. [Mallinckrodt Baker, 2000]). *p*-TSA was produced by Davos Chemical Corporation and RIT-Chem Company, Inc. in 1998 (U.S. EPA, 1999a, 2000), and by Unitex Chemical Co., Greensboro, NC (SRI, 1995; cited by HSDB, 2001a).

3.0 Production Processes

Chattaway (1905, cited by Budavari, 1996) reports the formation of chloramine-T through *p*-TSA. Chloramine-T is obtained from the reaction of *p*-TSA treated with sodium hypochlorite, chloramine B, $C_6H_5SO_2NClCa$, halazone, $HOCC_6H_4SO_2NCl_2$, and *N*-chloro-*N*-methyl-*p*-toluenesulfonamide (Nelson, 1985).

Chloramine-T (trihydrate form) may also be formed by a reaction of ammonia and *p*-toluenesulfochloride under pressure (Lewis, 1993). The latter is reacted with sodium hypochlorite in the presence of an alkali. Chloramine-T is produced from this reaction by crystallization.

p-TSA is formed by reaction of *p*-toluenesulfonyl chloride with ammonia (OECD, 1994). All processes are in a closed system except for drying and packaging. *p*-TSA is also formed by amination of *p*-toluene sulfochloride (Lewis, 1993).

4.0 Production and Import Volumes

Production and import volumes for chloramine-T were not located. The United States aggregated production volumes for chloramine-T were reported to be between 10,000 and 500,000 lbs. in 1998. Total aggregated production of *p*-TSA was reported between one and ten million lbs. for the same year. Davos Chemical Corporation and RIT-Chemical Company, Inc. each produce greater than 10,000 lbs *p*-TSA/year (U.S. EPA, 2000). In Japan, production volumes of *p*-TSA were about 1700 and 1000 metric tons for 1985 and 1991, respectively (OECD, 1994).



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